

Remarks

In the Response to Restriction Requirement dated 10 January 2006, applicants elected without traverse to have claims 1-11 (Group I) examined on the merits. Claim 12 (Group II) was therefore withdrawn from consideration. In the Office Action mailed on 4 April 2006 under Ex parte Quayle, the Examiner indicated that claims 12-13 had been canceled. To perfect the record, claim 12 is canceled herein. Accordingly, claims 1-11 are pending in the present application.

Rejections Pursuant to 35 U.S.C. §112, First Paragraph

In the Office Action, claims 1-12 were rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. In support of that rejection, the Examiner asserted that the claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Examiner further asserted that the original specification fails to disclose "7-Nitro-benz-2,1,3-oxadiazol" and that the Amendment filed on 3 October 2005 appears to introduce new matter.

In the Office Action dated 28 June 2005, the disclosure was objected to because the term NBD-amine was not adequately defined, and appropriate correction was required. On 28 September 2005, applicants amended paragraph [0009] of the specification, wherein "NBD" was defined as --7-Nitro-benz-2,1,3-oxadiazol--. On 4 April 2006, an Office Action under Ex parte Quayle was mailed wherein the Examiner announced that claims 1-11 were allowed, and that the application is in condition for allowance except for the fact that the disclosure lacks a Brief Description of the Drawings. This formal matter was addressed in the Amendment filed on 25 April 2006. There was no objection to the addition of "Nitro-benz-2,1,3-oxadiazol" in the Ex parte Quayle action.

In response to the Office Action mailed 25 July 2006, applicants have amended paragraph [0009] in order to clarify that NBD is the abbreviated form of "7-nitrobenzo-2-oxa-1,3-diazol". Attached hereto as Exhibit A are the first page of three (3) publications as examples, each illustrating that "7-nitrobenzo-2-oxa-1,3-diazol" is known in the art as "NBD". The composition shown in Fig. 1 of the present application is the N-oxide of the N,N-Di(hydroxyethyl)-NBD amine. Also, paragraph [0024] of the present application recites, as an example, "the N-oxide of the NBD-amine was prepared according to P.B. Ghosh, M.W. Whitehouse, J. Med. Chem., 11, 305-311 (1968)." No new matter has been added.

In view of the present Amendment, applicants submit that the present application is in compliance with the statute and respectfully request that the rejection be withdrawn.

Rejection Pursuant to 35 U.S.C. §102

Also in the Office Action, claim 12 was rejected under 35 U.S.C. §102(b) as being anticipated by Ghosh et al. (U.S. Pat. No. 4,358,595), Hones (U.S. Pat. No. 5,334,508) or Albarella (U.S. Pat. No. 6,872,573). Claim 12 is canceled herein, thereby mooted the instant rejection. Applicants respectfully request that the rejection be withdrawn.

Conclusion

Applicants have filed a complete response to the outstanding Office Action and respectfully submit that, in view of the above amendments and remarks, the application is in condition for allowance. The Examiner is encouraged to contact the undersigned to resolve efficiently any formal matters or to discuss any aspects of the application or of this response. Otherwise, early notification of allowable subject matter is respectfully solicited.

Respectfully submitted,

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Exhibit A

Biophysical Characterization of the Cocaine Binding Pocket in the Serotonin Transporter Using a Fluorescent Cocaine Analogue as a Molecular Reporter*

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To explore the biophysical properties of the binding site for cocaine and related compounds in the serotonin transporter SERT, a high affinity cocaine analogue (3 β -(4-methylphenyl)tropane-2 β -carboxylic acid *N*-(*N*-methyl-*N*-(4-nitrobenzo-2-oxa-1,3-diazol-7-yl)ethanolamine ester hydrochloride (RTI-233; K_f = 14 nM) that contained the environmentally sensitive fluorescent moiety 7-nitrobenzo-2-oxa-1,3-diazole (NBD) was synthesized. Specific binding of RTI-233 to the rat serotonin transporter, purified from Sf-9 insect cells, was demonstrated by the competitive inhibition of fluorescence using excess serotonin, citalopram, or RTI-55 (2 β -carbomethoxy-3 β -(4-iodophenyl)tropane). Moreover, specific binding was evidenced by measurement of steady-state fluorescence anisotropy, showing constrained mobility of bound RTI-233 relative to RTI-233 free in solution. The fluorescence of bound RTI-233 displayed an emission maximum (λ_{max}) of 532 nm, corresponding to a 4-nm blue shift as compared with the λ_{max} of RTI-233 in aqueous solution and corresponding to the λ_{max} of RTI-233 in 80% dioxane. Collisional quenching experiments revealed that the aqueous quencher potassium iodide was able to quench the fluorescence of RTI-233 in the binding pocket (K_{SV} = 1.7 M⁻¹), although not to the same extent as free RTI-233 (K_{SV} = 7.2 M⁻¹). Conversely, the hydrophobic quencher 2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO) quenched the fluorescence of bound RTI-233 more efficiently than free RTI-233. These data are consistent with a highly hydrophobic microenvironment in the binding pocket for cocaine-like uptake inhibitors. However, in contrast to what has been observed for small-molecule binding sites in, for example, G protein-coupled receptors, the bound cocaine analogue was still accessible for aqueous quenching and, thus, partially exposed to solvent.

Cocaine is one of the most widely abused psychostimulants, causing major medical and socioeconomic problems (1). Currently, there is no effective treatment against cocaine addiction available; therefore, clarifying the molecular mechanisms underlying the psychostimulatory effects and addictive properties of cocaine should prove critical for potential development of future therapeutic strategies. Cocaine and related drugs act by inhibiting clearance of released monoamine neurotransmitters from the synaptic cleft (2–4). This clearance of monoamines occurs via three distinct but highly homologous monoamine transporters, the serotonin transporter (SERT), the dopamine transporter (DAT), and the norepinephrine transporter (NET) (2–4). Cocaine binds with high affinity to all three transporters and is generally believed to act as a competitive blocker of substrate translocation (4, 5). Several studies have provided evidence that inhibition of the DAT is the predominant mechanism behind the stimulatory effects and addictive properties of cocaine (6–8). However, this hypothesis has been challenged by recent studies on mice in which the DAT gene has been deleted (1). Despite the absence of the DAT gene, it was surprisingly observed that these mice self-administered cocaine, indicating a possible important role of also the SERT and NET (9, 10).

The SERT belongs together with DAT and NET to a family of Na⁺/Cl⁻-dependent solute carriers that are characterized functionally by their dependence on the presence of Na⁺ and Cl⁻ in the extracellular fluid (3, 11). All Na⁺/Cl⁻-dependent carriers are believed to share a common topology characterized by the presence of 12 transmembrane segments connected by alternating extracellular and intracellular loops with an intracellular location of the N and C terminus (3, 4, 11). Despite intense efforts, including many mutagenesis studies (12–18) and studies using photoaffinity labeling (19), surprisingly little is known about the binding site for cocaine-like substances in the monoamine transporters. Although cysteine-scanning mutagenesis of transmembrane segment 3 in the SERT has suggested that two residues (Ile-173 and Tyr-176) in the middle of the transmembrane segment could be in close proximity to the cocaine binding site (20), no direct contact sites have been established between cocaine and specific transporter residues.

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‡ The abbreviations used are: SERT, serotonin transporter; rSERT, rat SERT; NET, norepinephrine transporter; DAT, dopamine transporter; NBD, 7-nitrobenzo-2-oxa-1,3-diazole; 5-HT, 5-hydroxytryptamine; TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl; RTI55, 2 β -carbomethoxy-3 β -(4-iodophenyl)tropane; RTI-233, 3 β -(4-methylphenyl)tropane-2 β -carboxylic acid *N*-(*N*-methyl-*N*-(4-nitrobenzo-2-oxa-1,3-diazol-7-yl)ethanolamine ester).

SYNTHESIS OF FLUORESCENT AND RADIOLABELED ANALOGUES OF PHOSPHATIDIC ACID

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Procedures for the synthesis of fluorescent and radiolabeled analogues of phosphatidic acid are described. The fluorophore 7-nitrobenzo-2-oxa-1,3-diazole (NBD) was coupled to 6-aminocaproic acid and 12-aminododecanoic acid by reaction of NBD-chloride with the amino acids under mild alkaline conditions at room temperature. 1,2-Dioleoyl-*sn*-[U-¹⁴C]glycerol 3-phosphate was prepared by acylation of *sn*-[U-¹⁴C]glycerol 3-phosphate with oleic acid anhydride using dimethylaminopyridine as the catalyst. This compound was converted to 1-oleoyl-*sn*-[U-¹⁴C]glycerol 3-phosphate by hydrolysis with phospholipase A₂. The lysophosphatidic acid was reacylated with NBD-aminocaproyl imidazole or NBD-aminododecanoyl imidazole to form the fluorescent, radiolabeled analogue of phosphatidic acid. Fluorescent, non-radiolabeled analogues of phosphatidic acid were prepared by phospholipase D hydrolysis of fluorescent phosphatidylcholine.

Keywords: 7-nitrobenzo-2-oxa-1,3-diazole; fatty acid; phosphatidic acid; phospholipids; acylation reactions

Introduction

Phosphatidic acid is an important intermediate for the biosynthesis of glycerolipids in mammalian cells. In order to follow the intracellular localization and metabolism of phosphatidic acid, we have prepared fluorescent analogues where the *sn*-2 position contains NBD linked to either 6-aminocaproic acid or 12-aminododecanoic acid. These fluorescent phosphatidic acids are taken up by mammalian cells, transported to intracellular sites of lipid biosynthesis and converted to end products of lipid metabolism [1-3].

NBD-labeled phosphatidic acids are most easily obtained by phospholipase D hydrolysis of commercially available NBD-labeled phosphatidylcholine [1]. However, for part of our investigations it was necessary to prepare phosphatidic acid with

Abbreviations: NBD, 7-nitrobenzo-2-oxa-1,3-diazole; NBD-Cl, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole; NMR, nuclear magnetic resonance; Pyr, pyridinium ion; TLC, thin-layer chromatography.

The invariant chain forms complexes with class II major histocompatibility complex molecules and antigenic peptides "in vivo"

(antigen presentation)

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ABSTRACT The binding of a chicken ovalbumin peptide (residues 323-339), Ova-(323-339), to I-A^b molecules was investigated *in vitro* and *in vivo*. By using antigenic peptides labeled either with a hapten or with fluorescein, complexes formed *in vitro* between I-A^b and antigenic peptides were detected by Western blot analysis with an antibody recognizing the hapten 7-nitrobenzo-2-oxa-1,3-diazole and by scanning gels for fluorescence emitted by fluoresceinated peptide. Both techniques reveal that Ova-(323-339) binds not only to I-A^b α/β heterodimers and separated α and β chains but also to complexes of higher molecular mass. Additional analysis shows that one of these additional complexes contains I-A^b heterodimers, antigenic peptides, and also invariant chain. To explore the physiological role of these complexes, cells were incubated with haptenated peptide and the I-A^b-peptide complexes formed *in vivo* were purified by affinity chromatography using hapten-specific antibody. The complexes formed migrate with a significantly higher apparent molecular mass than the α/β heterodimers. A band at 180 kDa contained the α/β heterodimer, the antigenic peptide, and the invariant chain. These results show that *in vivo* high molecular mass complexes formed by the I-A^b heterodimer and the invariant chain bind antigenic peptides.

During the last decade, accumulated data have shown that a key role in immune recognition is played by a trimolecular complex formed by the T-cell receptor, major histocompatibility complex (MHC) molecules, and immunogenic peptides. Whereas B cells recognize antigens in their native form through their specific receptors, immunoglobulins, T cells do not (1-3). T cells recognize immunogenic peptides associated with syngeneic restriction MHC molecules. These peptides arise from the proteolytic degradation of protein antigens inside acidic compartments of antigen-presenting cells (4-7). A molecular model for this recognition has been delineated from experiments using antigenic peptides specifically bound to isolated MHC molecules to induce T-cell responses (8-11). This immune recognition requires the selective binding of peptides to MHC molecules. Differences among the various alleles of class II MHC molecules in their ability to bind antigenic peptides account for the molecular basis of MHC gene control of the immune response (12, 13).

Beside the major role of MHC molecules in recognition of antigenic peptides, several reports suggest the involvement of an additional protein: the invariant (Ii) chain. The highly polymorphic class II MHC molecules consist of two noncovalently linked glycoproteins, the α and β chains of apparent molecular masses 34 and 29 kDa. Inside the cells, α/β heterodimers are found to be associated with a third nonpolymorphic chain with various apparent molecular masses

among which the prominent ones are of 31 and 41 kDa (14, 15). The Ii chain is encoded by a single-copy gene unlinked to the MHC (16) but exists in multiple forms resulting from a number of events ranging from alternate initiation of translation to post-translational modifications (17-21). Despite extensive studies, the function of the Ii chain has remained unclear. It has been suggested that Ii chain is involved in the assembly and transport of class II MHC molecules (16, 22, 23) and in the antigen processing and presentation (24, 25). A widely accepted view is that the Ii chain binds to the antigen binding site, protecting it from binding endogenous peptides until encountering antigenic peptides in acidic compartments where Ii is released from class II MHC (26-28). No evidence has been given previously for the formation of a tetrameric complex involving α , β , and Ii chains and antigenic peptide.

In the present study, we investigated the biochemical characteristics of the complexes containing class II MHC molecules that bind antigenic peptides *in vitro* and *in vivo*. Experiments described in this paper demonstrate that the chicken ovalbumin peptide (residues 323-339) [Ova-(323-339)] binds to various molecular complexes, all involving I-A^b molecules but of higher apparent molecular mass than the α/β heterodimers. By using Western blot analysis with monoclonal antibody (mAb) ANO9, that recognizes the hapten 7-nitrobenzo-2-oxa-1,3-diazole (NBD) (29), we were able to detect *in vitro* specific binding of NBD-conjugated Ova-(323-339)-Tyr [NBD-Ova-(323-339)-Tyr] not only to I-A^b heterodimers but also to complexes of higher molecular mass. Fluorescence scanning of proteins separated on SDS/PAGE under nonreducing conditions confirmed these results and revealed binding of fluorescein isothiocyanate-conjugated Ova-(323-339) [F-Ova-(323-339)] to I-A^b conformers with apparent molecular masses of 55 and 64 kDa, as described (30, 31). Two additional molecular species of 105 and 180 kDa appear even more effective in binding the antigenic peptide. Further characterization by Western blot analysis of these molecular complexes indicates that the 180-kDa complex is composed of I-A^b heterodimers, antigenic peptide, and also an Ii chain. Furthermore, when mAb ANO9 was used to purify I-A^b-peptide complexes formed *in vivo*, it was found that the high molecular mass complexes, rather than the α/β heterodimers alone, bind antigenic peptides *in vivo*. Thus these data demonstrate that the Ii chain is directly involved *in vivo* in a multimolecular complex with the class II MHC molecules and antigenic peptides.

Abbreviations: MHC, major histocompatibility complex; Ii chain, invariant chain; Ova-(323-339), synthetic peptide representing amino acids 323-339 of chicken ovalbumin; F-Ova-(323-339), Ova-(323-339) labeled at its N terminus with fluorescein isothiocyanate; NBD, 7-nitrobenzo-2-oxa-1,3-diazole; NBD-Ova-(323-339)-Tyr, Ova-(323-339) with a tyrosine at position 340 and labeled at its N terminus with NBD; mAb, monoclonal antibody.

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